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GENETIC TOXICITY EVALUATION OF 1, 3, 3-TRINITROAZETIDINE

VOLUME IV: SUMMARY REPORT ON THE GENOTOXICITY OF TNAZ

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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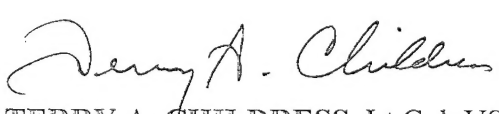

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In this study, 1,3,3-trinitroazetidine (TNAZ), a potential component of Air Force explosive formulations, was examined for mutagenic and clastogenic activity. The mutagenic activity of TNAZ was evaluated by both the *Salmonella* Microsome Reverse Mutation Assay and the Chinese Hamster Ovary Cell/Hypoxanthine-Guanine Phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay. TNAZ, in the presence and absence of S-9 microsomal enzymes, failed to induce histidine reversion (his- to his+) by base changes or frameshift mutation in the genome of five strains of *Salmonella typhimurium* bacterial. Furthermore, TNAZ, with and without S-9 metabolic activation, failed to induce a significant increase in mutant colonies at the HGPRT locus in cultured CHO cells. Additional independent confirmatory Ames and CHO/HGPRT assays indicated TNAZ was non-mutagenic. TNAZ was also examined for its effect on the chromosomes and mitotic apparatus of albino Swiss mice bone marrow cells by measuring micronuclei formation in maturing erythrocytes. The mouse bone marrow micronucleus test measures the clastogenic potential of chemicals *in vivo*. The number of micronucleated cells in the maturing erythrocytes in the bone marrow cells of mice was not increased in the presence of TNAZ but was increased greater than 30 fold by mitomycin C. In conclusion, under the test systems and conditions employed in this study, TNAZ is considered non-mutagenic and non-clastogenic.

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PREFACE

1,3,3-Trinitroazetidine (TNAZ) (CAS No. 97645-24-4) is a highly energetic castable explosive that is being considered by the Department of Defense for military and space applications. As a candidate replacement for select explosives, toxicity information is needed. A comprehensive literature search indicated that no information was available on the mutagenic potential of TNAZ. ManTech Environmental initiated a battery of three short-term assays that were utilized to assess the mutagenic and clastogenic potential of TNAZ. Protocols for these assays were in conformance with the Environmental Protection Agency's (Toxic Substances Control Act) Health Effects Testing Guidelines, 40 CFR, Part 798 (7-1-90 edition).

This document, Volume IV of IV, serves as a summary report presenting the pertinent findings of the three assays that were described in Volumes I through III:

Volume I	results of the *salmonella typhimurium* reverse mutation assay (Ames assay),
Volume II	results of the mouse bone marrow micronucleus test, and
Volume III	results of gene mutation at the HGPRT locus in cultured Chinese hamster ovary cells

The research described herein began in July 1992 and was completed in December 1993 by the Toxikon Corporation, Woburn, MA, under a subcontract to ManTech Environmental Technology Inc., Toxic Hazards Research Unit (THRU), and was coordinated by Darol E. Dodd, Ph.D., THRU Laboratory Director. This work was sponsored by the Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory, and was performed under Department of the Air Force Contract No. F33615-90-C-0532 (Study No. F19). Lt Col James N. McDougal and Lt Col Terry A. Childress, respectively, served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

The Toxikon Corporation has provided written permission to reprint this report herein.

ABSTRACT

In this study, 1,3,3-Trinitroazetidine (TNAZ), a potential component of Air Force explosive formulations, was examined for mutagenic and clastogenic activity. The mutagenic activity of TNAZ was evaluated by both the Salmonella Microsome Reverse Mutation Assay and the Chinese Hamster Ovary Cell/Hypoxanthine-Guanine Phosphoribosyl Transferase Forward Mutation Assay. TNAZ, in the presence and absence of S-9 microsomal enzymes, failed to induce histidine reversion (his- to his+) by base changes or frameshift mutations in the genone of five strains of Salmonella typhimurium bacteria. Furthermore, TNAZ, with and without S-9 metabolic activation, failed to induce a significant increase in mutant colonies at the HGPRT locus in cultured Chinese Hamster Ovary cells. Additional independent confirmatory Ames and CHO/HGPRT assays indicated TNAZ was non-mutagenic. TNAZ was also examined for its effect on the chromosomes and mitotic apparatus of albino swiss mice bone marrow cells by measuring micronuclei formation in maturing erythrocytes. The mouse bone marrow micronucleus test measures the clastogenic potential of chemicals *in vivo*. The number of micronucleated cells in the maturing erythrocytes in the bone marrow cells of mice was not increased in the presence of TNAZ but was increased greater than 30 fold by mitomycin C. In conclusion, under the test systems and conditions employed in this study TNAZ is considered non-mutagenic and non-clastogenic.

INTRODUCTION

The Air Force Armament Laboratory is investigating new explosive ingredients at their High Explosive Research and Development Facility (Borman, 1994). One compound that shows promise for replacing octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), a highly energetic and castable explosive, is 1,3,3-trinitroazetidine (TNAZ). Acute toxicity studies with TNAZ on laboratory animals indicate that it is nontoxic dermally, presents no sensitization potential, causes transient eye irritation, and is moderately toxic orally. However, the cellular and molecular interactions of TNAZ are still largely unknown. TNAZ may result in direct and/or indirect damage to DNA, and may initiate events (mutations) which may cause cell transformation and eventually lead to cancer. The structurally related compound 1-nitroazetidine has been demonstrated to be converted to 1-nitrosoazetidine which is a known carcinogen (Lijinsky et al., 1984). Genetic toxicology testing with TNAZ is essential since it may itself be converted or metabolize to a carcinogenic nitroso derivative. Based on the hypothesis that carcinogens are initially mutagens (some causing base pair substitutions and others causing frameshift mutations) and that carcinogenesis is the result of somatic mutations, Ames and colleagues experimented with microbial systems as an initial screen to detect possible carcinogens. The Ames assay system developed was shown to respond to known carcinogens (Ames et al., 1975). The utility of this microbial test system in detecting carcinogens/mutagens has been demonstrated extensively by a number of different laboratories (DeSerres and Shelby, 1979; McCann and Ames, 1976; Maron and Ames, 1983).

The basis of the Ames assay is a reverse-mutation from the nutrient-dependent strain (his-) to a "wild-type" strain (his+) capable of sustaining itself on minimal medium (Ames et al., 1975). The reverse mutation assays, using organisms with mutations at an easily detected locus, provide quite a small, specific and selective target site for the chemical to act on. On the other hand, forward mutation assays (eg., Chinese Hamster Ovary/Hypoxanthine Guanine Phosphoribosyl Transferase Assay [CHO/HGPRT]) present a larger genetic target for the chemical to act upon, with mutations occuring at several loci within one gene or being spread over several genes and with the relatively easy identification of a new phenotype (Li et al., 1988).

The Ames reverse mutation assay and the CHO/HGPRT assays are based on reactions related to the direct alteration at a specific locus on the DNA. The segregation of chromosomes is dependent upon an intact spindle-fiber production for mitosis, the molecular target and essential component being tubulin (Pickett-Heaps et al., 1982). Thus, tests based on point mutations would not reveal chemical-induced chromosomal malsegregation associated with tubulin during meiosis and mitosis. To test for the effects of a chemical on the chromosomes and mitotic apparatus the rodent micronucleus assay has been employed (Cole et al., 1981; Heddle et al., 1983, 1984; Kliesch et al., 1981; Matter and Schmid,

1971; Schmid, 1975; Schmid, 1976; Cihak, 1979).

The purpose of this study is to evaluate the mutagenic properties of TNAZ in both bacterial and mammalian cell systems. Furthermore, the *in vivo* clastogenic effects of TNAZ were examined utilizing the mouse bone marrrow micronucleus test.

MATERIALS AND METHODS

Trinitroazetidine was provided by Eglin Air Force Base, Eglin AFB, Florida. TNAZ has a structural formula of $C_3N_4H_4O_6$ (CAS No. 97645-24-4) and is a white granular solid with a particle size of approximately 100 microns. It is synthesized with 98-99% purity and has a crystal denisity of 1.84. Results of solubility tests (R. Nolan, Elgin AFB, FL) indicate that TNAZ is nonsoluble in water and in corn oil, slightly (approximately 10%) soluble in saline, and 100% soluble in dimethylsulfoxide or acetone.

Ames Assay:

The Ames assay detects base pair mutations and/or frameshift mutations (Ames et al., 1975). A Range Finding Assay was initially performed with TNAZ on the Salmonella typhimurium TA100 bacterial strain in the presence and absence of S9 microsomes in order to determine levels at which TNAZ exhibited toxicity and to determine what concentrations of TNAZ would be utilized in the Ames Mutagenicity assay. The S9 microsome mixture was obtained from rat liver homogenate obtained from Aroclor^R 1254 treated Sprague Dawley rats (Microbiological Associates, Rockville, MD). The range finding assay was conducted with TNAZ concentrations of 10000, 1000, 100, 10, 1, and 0.1 ug/plate.

The Reverse Mutation Assay was performed on histidine requiring strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537 and TA1538) (Ames et al., 1975). Each bacterial strain was exposed to TNAZ with and without exposure to S9 microsome mixture. The positive control article requiring metabolic activation was 2-aminoanthracene in all strains. Positive control articles not requiring metabolic activation are strain specific. The positive control for TA98, TA100, TA1535, TA1537 and TA1538 are 2-nitrofluorene (10 ug/ml), sodium azide (100 ug/ml), sodium azide (5 ug/ml), 9-aminoacridine (800 ug/ml) and 2-nitrofluorene (10 ug/ml) respectively.

All strains were treated with six concentrations of TNAZ. After a 20 minute incubation at 37^{o}C, tubes were vortexed and poured onto minimal glucose agar plates. Top agar, supplemented with 0.5 mM histidine and 0.5 mM biotin per 1.0 ml of agar, was used as the overlay. Plates were incubated at 37$\pm$2^{o}C for 48-65 hours, checked for uniform background lawns, and revertant colonies counted.

HGPRT Gene Mutation Assay:

The CHO/HGPRT assay was used to evaluate the mutagenic potential of TNAZ by its ability to induce forward mutations at

the HGPRT locus in Chinese Hamster Ovary cells. This assay system utilizes toxic purine analogs to select for resistant cells that are deficient in the purine salvage enzyme HGPRT (Li et al., 1988). The CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetalus griseus*) and was obtained from the American Type Culture Collection, Rockville, MD. CHO cells were routinely propagated in complete Ham's F-12 culture medium (Ham's F-12 nutrient medium supplemented with 10% heat inactivated fetal bovine serum, 2mM L-glutamine, 50 units/mL penicillin and 50 ug/mL streptomycin). Medium used for reducing the spontaneous frequency of HGPRT$^-$ mutants prior to the initiation of the study consisted of complete Ham's F12 medium supplemented with 5.0×10^{-6}M thymidine, 1.0×10^{-5} hypoxanthine (Hx), 2.0×10^{-4}M glycine, and 3.2×10^{-6}M aminopterin. Selection medium was prepared from complete medium that lacked hypoxanthine, but contained 10 ug/mL 6-thioguanine (TG) and 5% fetal bovine serum.

In the HGPRT/CHO assay, TNAZ was dissolved at a concentration of 5.0 mg/mL in a 0.5% DMSO solution in Ham's F-12 medium. The negative control used was serum free Ham's F-12. The final concentration of DMSO (0.5%) in the growth medium was assayed as the solvent control to determine any effects on survival or mutation caused by the solvent alone. The positive controls, 4-nitroquinoline-1-oxide (4-NQ), was dosed at a concentration of 0.03 ug/mL in the non-activated system and dimethylnitrosamine (DMN) was used at a concentration of 0.3 uL/mL in the activated system.

The S9 microsomal fraction, prepared from Sprague-Dawley rat livers induced with Aroclor 1254, was added to a medium·cofactor mixture. The medium·cofactor mixture consisted of 10% 10X isocitrate·NADP cofactors and 90% incomplete Hx-free, serum-free medium to give the following concentrations in the cell culture: 4.5 mg/mL isocitrate (trisodium salt), 2.4 mg/mL NADP (disodium salt), and 20 uL/mL S9 fraction.

The CHO/HGPRT assay was performed as follows: Duplicate cultures seeded with 5×10^5 cells/flask were used at each dose level. The cells were treated with seven concentrations of TNAZ and one dose of positive, negative and solvent control in both the activated and non-activated systems. In the activated system, cells were exposed to TNAZ for 5 hours and in the absence of S-9 microsomal activation, cells were exposed for 16 hours. The cells were then washed with PBS and refed with complete Ham's F-12 medium. After five days, the cells were harvested and reseeded in selection medium at 2×10^5 cells per 100 mm dish. Five dishes were plated per dose level for a total of 1×10^6 cells. Concurrently, 200 cells per 100 mm dish were seeded in complete medium for the Parallel Cloning Efficiency Assay. The cultures were then incubated for six days without disturbing the dishes to minimize the formation of satellite colonies. The colonies were then washed with PBS, fixed, stained and counted for cloning efficiency, and mutant selection. The average number of clones from the triplicate dishes was calculated and expressed as the Parallel Cloning Efficiency. The number of TG-resistant mutants for 1×10^6 cells seeded at each dose level was calculated by totaling the number of mutants from the five replicate dishes.

Based on the PCE, the number of TG-resistant mutants per 1 x 10^6 surviving cells was calculated for each dose level.

Micronucleus Assay:

The purpose of the micronucleus assay is to evaluate the clastogenic activity of a chemical and/or its metabolites via its ability to induce micronuclei formation in maturing erythrocytes of mice (Kliesch et al., 1981). Albino Swiss mice (*Mus musculus*), male and female, were obtained from the Charles River Breeding Laboratories, Wilmington, MA. At the start of the study, the animals were 7-12 weeks of age and $\geq$ 24 grams. Animals were randomized into treatment and control groups and identified by ear punch.

TNAZ was suspended in corn oil for administration. Corn oil served as the negative control and mitomycin C served as the positive control. TNAZ and the negative control were administered as three daily doses. The positive control substance was administered as a single dose. TNAZ and controls were dosed at a rate of 40 ml/kg. The dose levels for TNAZ in the Micronucleus Assay were selected based on the results of the Range Finding Assay. Four treatment groups of three animals per sex were selected for dosing in the Range Finding Assay. The test doses employed were 500, 100, 10, and 1.0 mg/kg per day for 3 days. Clinical observations were conducted daily for 72 hours during the period of dosing. Based on the results of the Range Finding Assay the dose levels selected for the Micronucleus Assay were 40, 20, 10, 5, and 1.0 mg/kg.

For the Micronucleus assay, animals were randomized and placed into treatment groups consisting of 5 males and 5 females. The test and control substances were dosed by intraperitoneal injection. At 72 hours, 5 males and 5 females were sacrificed from each TNAZ treated and negative control treated groups after receiving 3 single doses 24 hours apart. All animals in the positive control group were sacrificed 24 hours after a single dose administration.

At each sacrifice interval, bone marrow slides were prepared. Briefly, the animals were euthanized by cervical dislocation. Immediately after sacrifice, the femur was removed by appropriate surgical techniques. A 22g x 1" needle with a 1 cc syringe was used to flush fetal calf serum through the bone marrow cavity. The bone marrow was flushed onto a clean, pre-labeled microscope slide. A second slide, clean and pre-labeled, was inverted and placed flush to the first slide. Using a circular motion, the two slides were rubbed together until the bone marrow was evenly dispersed. The two slides were gently pulled apart and air dried. The slides were stained with SIGMA's "Accustain" Giemsa (1 part stock stain solution to 19 parts distilled water, by volume) for 5 minutes and differentiated in distilled water for 30 to 90 seconds.

A total of 1000 polychromatic erythrocytes per mouse were scored for the presence of micronuclei. The scored elements were the number of micronucleated cells, and not the number of micronuclei. The proportion of polychromatic erythrocytes to total erythrocytes was determined. The slides were scored blindly

in order to reduce possible bias associated with the analysis. The slides were coded using random numbers. Each test and control group was analyzed separately for male versus female animals utilizing a Student t-test to analyze for possible sex differences. The frequency of micronucleated PCEs (polychromatic erythrocytes) in each dose group was compared to that of the respective negative control substance, using analysis of variance (ANOVA) and Newman-Keuls Test for confirmation of pairwise comparisons.

RESULTS

AMES Assay:

TNAZ at various concentrations was incubated with the Salmonella typhimurium TA100 strain, in the presence and absence of microsomal enzymes, in order to select nontoxic concentrations for the evaluation of the mutagenic effect of TNAZ with 5 strains of Salmonella typhimurium bacteria. TNAZ exhibited toxicity at various concentrations as determined by both a reduction in the number of spontaneous revertants and by a clearing of the background lawn. TNAZ concentrations greater than 500 ug/plate resulted in a total reduction in the number of revertants/plate. TNAZ at a dose of 100 and 50 ug/ml caused a 36% and 2% reduction in the number of revertants, respectively.

The mutagenic potential of TNAZ was assessed with the Salmonella typhimurium bacterial strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations elucidated from the Range finding assay (500-0.5 ug/ml). The Reverse Mutation Assay, both in the presence and absence of S-9 microsomes, indicated that all concentrations of TNAZ tested failed to significantly increase the number of his- to his+ revertants (Tables 1 and 2). The positive control articles which consisted of direct-acting mutagens and mutagens that require metabolic transformation significantly increased the number of revertants relative to the negative control. All positive controls exhibited at least twice the number of colonies as the negative controls, demonstrating that the test system was functional with known mutagens. The results of this assay were confirmed through an independent Confirmatory Assay run on fresh sample (Results not shown).

HGPRT Assay:

The CHO/HGPRT was used to evaluate the mutagenic potential of TNAZ via its ability to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in mammalian cells. The Range Finding Assay demonstrated that of the doses tested, the higher dose concentrations of 5.00, 2.00, 1.00, and 0.500 mg/mL were cytotoxic in the activated system. Therefore, 0.500, 0.250, 0.125, 0.062, 0.031, 0.016 and 0.008 mg/mL doses were utilized in the activated Mutagenicity Assay. In the non-activated Mutagenicity Assay 0.250, 0.125, 0.062, 0.031, 0.016, 0.008 and 0.004 mg/mL doses were used.

The Mutagenicity Assay (Tables 3 and 4), both in the

presence and absence of microsomal S-9 liver enzyme, demonstrated that TNAZ failed to induce significantly increased numbers of mutant colonies in the CHO/HGPRT assay. The effect of TNAZ on the expression of mutant colonies was similar to untreated (negative) and solvent controls, whereas both positive controls exhibited an increased induction of mutant colonies (Tables 3 and 4). Dimethylnitrosamine increased the mean mutant frequency of CHO cells by 33.8 fold in the activated HGPRT mutagenicity assay, whereas, 4-nitroquinoline-1-oxide increased the mean mutant frequency by 26.8 fold in the non-activated system.

The Mutagenicity Assay was repeated as a Confirmatory Assay (Results not shown). These results demonstrated that TNAZ did not increase the number of mutants and is comparable to that of the negative control substance under the conditions utilized in this test system.

Micronucleus Assay:

Albino Swiss mice were inoculated intraperitoneally with various doses of TNAZ in order to select nonlethal doses for subsequent evaluation of the clastogenic effects of TNAZ on bone marrow erythrocytes. Immediately after dosing, death due to the toxicity of TNAZ was observed in the Range Finding assay with all the animals dosed at 500 mg/kg. Death was also observed in three out of six animals dosed at 100 mg/kg immediately after dosing. The remaining three animals at the 100 mg/kg dose exhibited tremors. At 10 mg/kg tremors were observed in all animals. However, at 10 and 1 mg/kg no deaths were observed. From the range finding assay a dose range of 40-1 mg/kg TNAZ was selected for subsequent testing in the micronucleus assay.

TNAZ (dissolved in corn oil) at doses of 40, 20, 10, 5, and 1 mg/kg failed to induce an increase in the number of micronucleated cells in the mouse micronucleus assay (Tables 5 and 6). Therefore, TNAZ is considered non-mutagenic under the test system and conditions employed in this study. Mitomycin C induced a significant 11.3 fold increase in the number of micronucleated cells, whereas the negative control did not lead to an increase in the number of micronucleated cells in the maturing erythrocytes of albino swiss mice bone marrow.

Each test and control group in the micronucleus assay was analyzed separately for male versus female animals utilizing a Student t-test to analyze for possible sex differences. No statistical significant differences were noted in the frequency of micronuclei between males and females. The frequency of micronucleated PCEs (polychromatic erythrocytes) in each dose group was compared to that of the respective negative control substance, using ANOVA (analysis of variance) and Newman-Keuls Test for confirmation of pairwise comparisons. There was a statistically significant increase in the number of micronucleated polychromatic erythrocytes in the positive control group compared to the negative control group, at $p \leq 0.05$. TNAZ did not produce a statistically significant dose related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the concentrations tested.

DISCUSSION

TNAZ is a high energy material that is currently being considered as an experimental explosive by the U.S. Air Force (Borman, 1994). TNAZ is more powerful than the two most widely used explosives HMX (High-Melting Explosive, tetranitrotetrazacycloctane) and RDX (Research Department Explosive, trinitrohexahydrotriazine) (Borman, 1994). Although TNT is still widely used militarily, there are major environmental problems in its manufacture and its explosive power is lower than that of TNAZ, HMX and RDX. An advantage of TNAZ over HMX and RDX, in addition to its higher energy content, is that it is melt-castable, so that large amounts of it can be made up in a liquid form and directly poured into shape charges (Borman, 1994). Shape charges are used to attack heavily armored targets such as tanks, where the explosive force must be focused in a particular direction to penetrate the armor. Toxicological information is needed to evaluate TNAZ for potential use as an explosive replacement. Genetic toxicity studies are especially essential since the structurally related compound 1-nitroazetidine has been shown to be converted to 1-nitrosoazetidine, a known carcinogen (Lijinsky et al., 1984). This information leads to the concern that TNAZ also might be converted or metabolized to corresponding nitroso derivatives that are carcinogenic. There are no literature citations on TNAZ to support or refute this proposal. Because the genotoxicity of a compound may reflect its carcinogenicity potential, three common assays were selected to investigate the genotoxicity potential of TNAZ.

A clear-cut indication of mutagenic potential may be exceedingly difficult to attain from one test, particularly for weak mutagens. The limitations of any individual *in vitro* or *in vivo* mutagenicity test include variability in sensitivity, the complexity of the organism and its genetics, and the different ways in which an agent may affect the DNA. To circumvent the limitations of individual tests, we evaluated the mutagenic activity of TNAZ with a battery of tests which included: the detection of gene mutation in bacteria, the induction *in vitro* of gene mutation in mammalian cells, and the *in vivo* rodent micronucleus assay.

The Salmonella typhimurium Reverse Mutation Assay (Ames Assay) evaluated the potential of TNAZ to induce histidine (his) reversion (his- to his+), caused by base changes or frameshift mutations in the genome of this organism. This direct plate incorporation assay conducted with five strains of Salmonella typhimurium was performed in the presence and absence of an exogenous mammalian activation system. The preincubation technique was used to enhance the sensitivity of the plate incorporation assay. The Reverse Mutation Assay determined that TNAZ was non-mutagenic.

Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and V-79 and CHO lines of Chinese hamster cells. In these cell lines the most commonly

used systems measure mutation at the thymidine kinase, HGPRT and Na+/K+ ATPase loci. The thymidine kinase and HGPRT mutational systems detect base pair mutations, frameshift mutations and small deletions; the Na+/K+ ATPase system detects base pair mutations only (Arlett and Cole, 1988). In this study, the results of the Salmonella typhimurium reverse mutation assay for non-mutagenicity of TNAZ were confirmed using the CHO/HGPRT system. The CHO/HGPRT assay demonstrated that TNAZ failed to induce increased forward mutations at the HGPRT locus in CHO cells, with a forward gene mutation being detected by a change in the enzymatic and functional activity of the HGPRT protein.

The mouse bone marrow micronucleus assay is an *in vivo* cytogenetic assay based on the observation that cells with broken chromosomes or impairments of the spindle apparatus often have disturbances in the distribution of chromatin during cell division (Kliesch et al., 1981). Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes which lag behind at the anaphase stage of cell division (Schmid, 1975). After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple nuclei in the cytoplasm (Schmid, 1975). The rodent micronucleus test has proven to be a valuable tool for elucidating the clastogenic activity of chemicals *in vivo* (Cole et al., 1981; Heddle et al., 1983; Kliesch et al., 1981; Matter and Schmid, 1971; Schmid, 1975; Schmid, 1976; Cihak, 1979). In this study, the micronucleus test demonstrated that TNAZ did not induce damage to the chromosomes or to the mitotic apparatus of the cell. These results suggest that TNAZ is non-clastogenic, since it failed to increase the number of micronuclei formed in erythrocytes of Albino Swiss mice.

Based on the results of this study 1, 3, 3-Trinitroazetidine is considered non-mutagenic in both the bacterial S. typhimurium assay and in the mammalian HGPRT/CHO assay. TNAZ also failed to elicit clastogenic activity with the erythrocytes of mouse bone marrow cells.

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TABLE 1. Reverse Mutation Assay Without Microsomal Activation

Revertants/Plate[a]

STRAIN	CONTROLS		TEST ARTICLE			
			Dose Levels (ug/plate)			
	Positive Control[b]	Negative Control[c]	500	50	5	0.5
TA98	160	34	0	30	33	32
	163	33	0	32	30	31
	175	34	0	31	34	34
MEAN	166.0	33.7	0	28.0	32.3	32.3
SD	7.9	0.6	0	1.0	2.1	1.5
TA100	319	135	0	126	120	130
	326	129	4	135	131	129
	343	143	1	128	130	125
MEAN	329.3	135.7	1.7	129.7	127.0	128.0
SD	12.3	7.0	2.1	4.7	6.1	2.6
TA1535	146	22	0	24	25	21
	155	24	0	22	20	23
	155	23	0	23	24	24
MEAN	152.0	23.0	0	23.0	23.0	22.7
SD	5.2	1.0	0	1.0	2.6	1.5
TA1537	104	12	0	10	13	10
	107	12	0	11	12	10
	105	13	0	11	13	9
MEAN	105.3	12.3	0	10.7	12.7	9.7
SD	1.5	0.6	0	0.6	0.6	0.6
TA1538	123	18	0	15	16	20
	127	19	0	19	17	18
	124	17	0	18	19	17
MEAN	124.7	18.0	0	17.3	17.3	18.3
SD	2.1	1.0	0	2.1	1.5	1.5

a All plates were dosed at 100 ul/plate
b The positive control used was sodium azide for strains TA-100 and TA-1535, 2-nitrofluorene for strain TA-98, and 9-aminoacridine for strain TA-1537
c The negative control used in the assay was 0.5% DMSO

TABLE 2. Reverse Mutation Assay With Microsomal Activation

Revertants/Plate[a]

	CONTROLS		TEST ARTICLE			
STRAIN	Positive Control[b]	Negative Control[c]	Dose Levels (ug/ml) 500	50	5.0	0.5
	171	41	0	42	43	41
TA98	176	40	0	39	41	43
	176	40	0	40	40	44
MEAN	174.3	40.3	0	40.3	41.3	42.7
SD	2.9	0.6	0	1.5	1.5	1.5
	426	177	14	185	174	175
TA100	404	188	12	176	176	180
	434	180	9	179	172	177
MEAN	421.3	181.7	11.7	180.0	174.0	177.3
SD	15.5	5.7	2.5	4.6	2.0	2.5
	183	28	0	27	25	27
TA1535	190	23	0	27	24	26
	189	26	0	26	28	27
MEAN	187.3	25.7	0	26.7	25.7	26.7
SD	3.8	2.5	0	0.6	2.1	0.6
	126	12	0	15	13	14
TA1537	119	16	0	14	15	12
	125	15	0	12	15	12
MEAN	123.3	14.3	0	13.7	14.3	12.7
SD	3.8	2.1	0	1.5	1.2	1.2
	128	18	0	20	21	21
TA1538	136	21	0	20	18	23
	126	19	0	19	18	19
MEAN	130.0	19.3	0	19.7	19.0	21.0
SD	5.3	1.5	0	0.6	1.7	2.0

[a] All plates were dosed at 100 ul/plate
[b] The positive control used was 2-aminoanthracene for all strains
[c] The negative control used in the assay was 0.5% DMSO

TABLE 3. HGPRT MUTAGENICITY ASSAY (ACTIVATED ASSAY)

TNAZ (mg/mL)	AVE. MUTANT COLONIES/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
0.500	0.0	29.0	14.5	0.00
0.250	0.4	146.0	73.0	2.74 ± 1.73
0.125	0.6	156.0	78.0	3.87 ± 1.64
0.062	0.6	150.0	75.0	4.00 ± 1.74
0.032	1.0	159.0	79.5	6.29 ± 2.88
0.016	0.8	165.0	82.5	4.85 ± 1.26
0.008	0.6	135.0	67.5	4.44 ± 1.92
NEGATIVE CONTROL	0.4	162.0	81.0	2.47 ± 1.87
SOLVENT CONTROL (0.5% DMSO)	0.6	170.0	85.0	3.53 ± 2.36
POSITIVE CONTROL DMN (0.3 uL/mL)	18.6	156.0	78.0	119.23 ± 11.9

TABLE 4. HGPRT MUTAGENICITY ASSAY (NON-ACTIVATED ASSAY)

TNAZ (mg/mL)	AVE. MUTANT COLONIES/DISH	AVERAGE SURVIVING COLONIES	AVERAGE % PLATING EFFICIENCY	MEAN MUTANT FREQUENCY PER $1x10^6$ SURVIVORS
0.250	0.0	0.0	0.0	0.0
0.125	0.0	0.0	0.0	0.0
0.062	0.0	0.0	0.0	0.0
0.032	0.8	172.0	86.0	4.65 ± 2.92
0.016	0.4	160.0	80.0	2.50 ± 1.60
0.008	0.6	155.0	77.5	3.87 ± 1.65
0.004	0.4	158.0	79.0	2.53 ± 2.57
NEGATIVE CONTROL	0.4	166.0	83.0	2.41 ± 1.52
SOLVENT CONTROl (0.25%)	0.6	138.0	69.0	4.35 ± 2.95
POSITIVE CONTROL 4-NQ(0.03 uL/mL)	17.0	146.0	73.0	116.44 ± 14.9

TABLE 5. EFFECTS OF TNAZ ON MICRONUCLEATED CELL FORMATION IN BONE MARROW EXTRACT SMEARS (MALES)

Animal #	TNAZ (mg/kg)	#RBC	PCE/ RBC	#MNC/ 1000 PCE	AVG # MNC ± SD
1	40	355	8.42	3	
2	40	348	5.76	3	
3	40	352	8.47	3	3.6 ± 0.80
4	40	372	8.12	4	
5	40	396	5.03	5	
11	20	390	7.81	5	
12	20	382	8.12	6	
13	20	400	7.74	5	4.6 ± 1.02
14	20	368	8.13	3	
15	20	382	7.87	4	
21	10	377	7.84	5	
22	10	418	4.75	5	
23	10	458	6.80	4	5.0 ± 0.63
24	10	340	5.89	5	
25	10	331	5.86	6	
31	5	377	10.79	4	
32	5	360	5.56	5	
33	5	363	8.27	4	4.2 ± 0.40
34	5	323	9.33	4	
35	5	344	5.80	4	
41	1	357	8.36	5	
42	1	370	5.41	4	
43	1	352	8.51	3	4.4 ± 0.80
44	1	350	7.44	5	
45	1	346	5.80	5	
51	Neg. Control	428	4.84	3	
52	Neg. Control	425	4.70	3	
53	Neg. Control	414	4.82	4	3.6 ± 0.80
54	Neg. Control	400	7.57	3	
55	Neg. Control	349	5.64	5	
61	Pos. Control	411	9.46	49	
62	Pos. Control	324	9.38	42	
63	Pos. Control	329	9.06	41	42.4 ± 3.38
64	Pos. Control	332	9.88	40	
65	Pos. Control	408	7.51	40	

PCE = polychromatic erythrocytes
RBC = red blood cells
MNC = micronucleated cells

TABLE 6. EFFECTS OF TNAZ ON MICRONUCLEATED CELL FORMATION IN BONE MARROW EXTRACT SMEARS (FEMALES)

Animal #	TNAZ (mg/kg)	#RBC	PCE/ RBC	#MNC/ 1000 PCE	AVG # MNC ± SD
6	40	346	8.55	4	
7	40	360	5.32	4	
8	40	360	5.57	6	4.4 ± 1.02
9	40	374	8.17	3	
10	40	400	7.47	5	
16	20	387	7.83	5	
17	20	461	8.32	5	
18	20	363	8.32	3	4.2 ± 0.75
19	20	351	8.67	4	
20	20	353	8.51	4	
26	10	357	8.43	4	
27	10	386	5.14	5	
28	10	357	8.53	3	4.0 ± 0.63
29	10	376	5.33	4	
30	10	347	8.66	4	
36	5	360	8.35	4	
37	5	360	5.59	3	
38	5	358	8.19	4	4.4 ± 1.02
39	5	350	5.72	5	
40	5	408	4.94	6	
46	1	330	9.02	4	
47	1	390	7.71	6	
48	1	345	8.80	4	4.8 ± 0.75
49	1	364	5.00	5	
50	1	395	5.06	5	
56	Neg. Control	364	4.48	4	
57	Neg. Control	394	7.63	4	
58	Neg. Control	378	5.34	3	3.6 ± 0.49
59	Neg. Control	393	7.80	4	
60	Neg. Control	358	8.42	3	
66	Pos. Control	370	5.50	38	
67	Pos. Control	360	8.27	42	
68	Pos. Control	358	9.30	44	38.8 ± 4.45
69	Pos. Control	395	5.07	39	
70	Pos. Control	386	5.14	31	

PCE = polychromatic erythrocytes
RBC = red blood cells
MNC = micronucleated cells

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